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13. ABSTRACT *(Maximum 200 Words)*

Hybrid transgenic/genetically obese mice were bred to evaluate the effects of obesity on mammary tumor development (MT). In the TGF-α/*Lep*^{ob} Incidence Study, *Lep*$^{+}$*Lep*$^{+}$ lean, *Lep*$^{+}$*Lep*^{ob} lean, and *Lep*^{ob}*Lep*^{ob} obese mice were followed. Obese mice weighed more than lean mice, but died prior to MT development. Heterozygous mice weighed more than homozygous lean mice, died younger, and had higher MT incidence. Within genotypes, mice with MT were heavier and had higher fat pad weights. WEIGHT-CYCLED TGF-α/*Lep*$^{+}$*Lep*^{ob} mice were food restricted at 50% of *ad libitum* for 3 wk periods followed by 3 wk of *ad libitum* feeding resulting in ~25% reduction in caloric intake. WEIGHT-CYCLED mice had decreased incidence and increased latency of MT compared to AD LIBITUM and PAIR-FED mice. TGF-α/*Lep*$^{+}$*Lep*$^{+}$ mice fed a high-fat diet were assigned to OBESITY-PRONE and OBESITY-RESISTANT groups. OBESITY-PRONE mice developed MT at a significantly earlier age than OBESITY-RESISTANT mice and CHOW mice. A second hybrid strain, TGF-α/*Lepr*^{db} has also been established. The *Lepr*^{db} Incidence Study has completed enrollment. As with the *Lep*^{ob} mice, obese mice are dying at young ages, and the TGF-α/*Lepr*$^{+}$*Lepr*^{db} lean mice weigh more than *Lepr*$^{+}$*Lepr*$^{+}$ lean mice. MT have been detected in both lean groups. All mice have been enrolled in the *Lepr* Diet-Induced Obesity protocol, and we have almost completed enrollment in the Weight-Cycled protocol.

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INTRODUCTION:

Breast cancer is the most frequently diagnosed cancer in women (1). One risk factor that has been proposed to play a role in the development of postmenopausal breast cancer is increased body weight and/or body mass index (BMI) (weight/height^2). Several reviews of the literature have shown that both case-control and prospective studies support this conclusion (2,3). A recent study of Italian women concluded that overweight accounted for 10.2% of postmenopausal breast cancer cases (4). Other studies have implicated weight gain as a risk factor for breast cancer development (5-8). However, higher body weight and weight gain in breast cancer development have not been supported by all studies. This may be partially attributable to technical factors. For example, conflicting results from human studies might be due to inaccurate recalled body weight. Also, whether weights from before or after menopause are used makes a difference in the conclusions drawn (9). There are other confounding factors such as the influences of ethnic and social backgrounds, and the potential for interactions with other risk factors.

Animal models are frequently used to study human diseases. Currently, the number of published studies investigating the role of obesity and mammary tumorigenesis in animals is limited. However, these studies (10-17) indicate that obese mice and rats have a higher incidence of either chemically-induced or spontaneous mammary tumors. Two additional publications using weight-cycled rats further implicate weight gain as an important factor in the development of chemically-induced mammary tumors (18,19). In dogs, lower body weight was protective against mammary tumor development (20), while obesity was reported to increase the risk of mammary tumors (21). Clearly, the role of these factors as potential risk factors in the etiology of human breast cancer is an important issue to resolve.

We have proposed that the role of body weight, BMI and weight gain in the development of breast cancer can be addressed systematically in a physiologically relevant animal model, *i.e.*, transgenic mice. Our hypothesis is that weight gain and the accompanying metabolic changes create a milieu conducive to enhanced development of oncogene-induced mammary tumors. We have developed a molecularly well-defined animal model to test this hypothesis. "Hybrid" obese-transgenic mice were produced by mating strains of genetically obese mice in which the molecular defect has been identified, *i.e.*, *Lep*^{ob} or *Lepr*^{db}, with a transgenic mouse line overexpressing TGF-α. This proto-oncogene has been implicated in the etiology of human breast cancer (22,23), and its presence in mice has been demonstrated to result in a 30% incidence of mammary tumors by 15-16 months of age (24). These hybrid mice are being used to systematically evaluate the role of body weight and weight gain in the development of mammary tumors in genetically obese, dietary obese, and lean mice.

BODY:

Due to the overlapping time sequences and the long-term nature of these experiments, I will describe each study individually. In general, the goals in the Statement of Work (Appendix A) have been met with some modifications that will be described were appropriate. Please note that results are presented as means ± sem in tables. In figures, error bars are not always included for clarity.

1- TGF-α/*Lep*^{ob} Strain Mice Incidence Study.

A total of 39 homozygous *Lep*$^{+}$*Lep*$^{+}$ lean mice, 41 heterozygous *Lep*$^{+}$*Lep*^{ob} lean mice, and 59 homozygous *Lep*^{ob}*Lep*^{ob} obese mice were enrolled in the incidence study (all mice carried the TGF-α gene). As indicated in the first year's report, we included heterozygous mice in this regimen when we found out that the mice carrying TGF-α would not lactate, and thus we could not use these mice for breeding purposes as originally planned. More obese than lean mice were enrolled in the study. This was due to the excess mortality of the obese mice. Mice were weighed weekly and assessed for the presence of mammary tumors (MT). Once MT were detected, their growth was monitored and the mice were killed when MT size exceeded 20 mm in length or if the location of the tumor interfered with the animal's well-being. Other criteria for euthanasia included excessive weight loss, unhealed skin lesions and/or other factors as determined by the animal care handlers. Mice that were still alive at 104 wk of age were euthanized. All mice are now dead in this protocol except for one homozygous obese mouse (81 wk of age).

The growth curves for the three genotypes of the TGF-α/*Lep*^{ob} strain mice are shown in Figure 1 (Appendix B). Body weights are presented by 4-week intervals. Body weights were statistically different at all time points among the three groups when analyzed by ANOVA. Post hoc analysis indicated that, as expected, the obese mice were heavier than the two lean groups. The two lean groups were not statistically different from each other by this analysis, but, when analyzed by a Student's "t" test, all comparisons between the two lean groups were significantly different (Figure 2, Appendix C). The obese mice died at a significantly younger age than did the lean mice, as shown in the survival curves (Figure 3, Appendix D). Interestingly, the heterozygous lean mice that weighed on average significantly more than the homozygous lean mice died significantly earlier than did the homozygous lean mice.

MT developed in 26 of 41 TGF-α/*Lep*$^{+}$*Lep*^{ob} lean mice between 48 and 104 wk of age and in 18 of 39 TGF-α/*Lep*$^{+}$*Lep*$^{+}$ mice between 65 and 104 wk of age. MT were detected at a younger age in the *Lep*$^{+}$*Lep*^{ob} compared to *Lep*$^{+}$*Lep*$^{+}$ lean mice, *i.e.*, 81.2 ± 3.1 vs 89.1 ± 3.0 ($p = 0.08$) wk of age. No MT were found in homozygous obese mice. This was not unexpected since the average age of death for the obese mice was 55.7 ± 2.0 wk of age, much earlier than when most MT were detected in the other two genotypes.

Final body weights of the *Lep*$^{+}$*Lep*^{ob} lean mice were found to be higher than the body weights of the *Lep*$^{+}$*Lep*$^{+}$ lean mice (Table 1, Appendix E). In addition, mice with MT were found to be heavier than those without MT. Body weights were corrected for the weight of MT, organs and other tumors by subtracting these values from final body weight and this was termed carcass

weight. A similar relationship was still found, *i.e.*, the mice with MT had heavier carcass weights compared to mice without MT. Mice with MT also had heavier combined retroperitoneal and parametrial fat pad weights than did mice without tumors (Table 1, Appendix E). There were no significant differences for MT number or burden (weight) between the two genotypes.

Blood samples have been obtained from most of the mice that were euthanized, and we are initiating analyses for leptin, triglycerides and cholesterol. Tissue samples have been delivered to Dr. Joseph Grande, co-investigator, a pathologist at Mayo Clinic. To date, 33 pathology reports have been received for the heterozygous mice and 15 for the homozygous lean mice. Results to date confirm that MT are adenocarcinomas. We have also obtained serum and tissue samples from nontransgenic mice of the three genotypes that were 13-14 months of age when euthanized to provide "control reference" data. Mice were kept alive longer than what was originally proposed because MT development seemed slower than expected from what was published.

2- TGF-α/*Lep*$^{+}$*Lep*^{ob} Mice in Weight-cycling Study.

TGF-α *Lep*$^{+}$*Lep*^{ob} mice were enrolled in the weight-cycling study. Due to the problems we had with housing the obese mice individually and the uncertainty at this time as to when and if they develop MT, we have not included them in this protocol. Three groups of mice are being used as described in the original protocol. The AD LIBITUM-FED group (n=30) was fed purified Diet C on an *ad libitum* basis. This diet is based on AIN93 recommendations for maintenance feeding of mice and rats (25). The WEIGHT-CYCLED group (n=30) was fed purified Diet D for 3 wk intervals at 50% of the intake of the AD LIBITUM-FED group. This diet is isocaloric with Diet C but has twice the protein, vitamin, mineral and fat content in order that the two groups of mice receive an equivalent amount of these nutrients. The major difference between the two diets is calories due to the amount of carbohydrate. Following each 3 wk food restriction period, the WEIGHT-CYCLED mice were then fed Diet C *ad libitum* for 3 wk. An additional control group (n=33) was chronically food restricted a mixture of Diet C and D (2:1) to match the intake of the WEIGHT-CYCLED mice for each 6 wk interval, *i.e.*, PAIR-FED. By necessity, this group was enrolled to follow the weight-cycled group. Food intake was determined daily and body weights were measured weekly. As described earlier, the mice were also monitored for the presence of MT and were euthanized due to tumor size or other factors. Mice in this protocol that were not dead by 80 wk of age were killed then because of the acceleration of MT development when they were fed the purified Diet C compared to chow (described below). At 80 wk of age, WEIGHT-CYCLED mice had completed 12 food restriction periods and one week of refeeding in the 12th cycle. WEIGHT-CYCLED mice that were killed at an earlier age were killed during refeeding. The WEIGHT-CYCLED mice killed prior to the termination of the experiment had failed to regain lost weight. All AD LIBITUM-FED mice are dead, as well as 21 of the WEIGHT-CYCLED mice and 17 of the PAIR-FED mice. WEIGHT-CYCLED MICE that are alive are 75 wk of age or older and live PAIR-FED mice are 72 wk or older (9/15/00).

Body weights for the mice over the course of the study are shown in Figure 4 (Appendix F). As can be seen, the general pattern for the first 6 cycles was that WEIGHT-CYLED mice lost substantial weight during the first week of restriction, then their body weights stabilized for the next two weeks. During the later cycles, the WEIGHT-CYCLED mice continued to lose additional small amounts of weight during the second and third weeks of food restriction. There

are still animals alive so the numbers are not complete for the last 8 weeks of the experiment. In all refeeding periods, when *ad libitum* feeding was instituted, the WEIGHT-CYCLED mice rapidly regained their lost weight. In most intervals, refeeding resulted in the WEIGHT-CYCLED mice attaining body weight levels of the AD LIBITUM-FED mice, particularly in the older mice.

Food intake calculations for each six week feeding interval indicated that AD LIBITUM-FED mice consumed more food that did mice in either WEIGHT-CYCLED or PAIR-FED groups (data not shown). Cumulative food intake for the dead mice was found to be 28% greater in AD LIBITUM-FED compared to WEIGHT-CYLCED and PAIR-FED mice (Figure 5, Appendix G). Final body weights were similar for all dead mice as shown in Figure 6 (Appendix H). As indicated, all of the AD LIBITUM-FED mice are dead and 77% of them had MT. In contrast, only one of the 21 dead WEIGHT-CYCLED mice had a MT, and this was detected at death and weighed only 0.063 g compared to average MT weight of 1.065 g for the AD LIBITUM-FED mice. For the PAIR-FED mice, 8 of 17 have been identified with MT, but some of these were detected when the mice were killed at 80 wk of age and were very small. We await confirmation of MT pathology and completion of calculations and statistical evaluations when the remaining weight-cycled and food restricted mice have died to finalize our conclusions of this experiment. However, it does appear that caloric restriction in the form of intermittent caloric deficit followed by *ad libitum* feeding resulted in increased latency and lower incidence of MT. This contrasted with results of a higher MT incidence in weight-cycled animals from a previous study using rats with chemically-induced MT. However, caloric restriction was not as great in that study and a high-fat diet was used.

As mentioned above, we found that the AD LIBITUM-FED mice in this protocol that were fed a purified diet based on AIN-93M recommendations (DIET C) developed MT at much earlier ages than did the mice of the same genotype, $Lep^{+}Lep^{ob}$, fed chow in the Incidence Study. This is demonstrated in Figure 7 (Appendix I) where curves for age of MT detection for the two groups are compared. The average age of MT detection was 63.1 ± 1.9 (n=23) weeks compared to 82.8 ± 3.2 (n=25) weeks for the AD LIBITUM-FED DIET C mice versus CHOW mice. The survival curves are shown in Figure 8 (Appendix J). The MT incidence rates were 77% versus 61% for the DIET C versus CHOW mice, respectively. Since the DIET C mice were only allowed to live until 80 wk of age, it is likely that if those without MT at 80 wk of age had been allowed to live longer that the incidence rate would have been even greater. Body weights at the time of MT detection were not different between the groups. Body weights at death were higher in the CHOW mice (35.89 ± 0.80g) compared to the AD LIBITUM-FED DIET C mice (31.83 ± 1.43 g) [p 0.014]. However, fat pad weights of the AD LIBITUM-FED DIET C mice (1.519 ± 0.047 g) were significantly greater than those of the CHOW mice (1.023 ± 0.022 g) [p <0.0001]. Completion of serum analyses may help to further identify differences between these two groups. However, it appears at this time that the presence and/or absence of some dietary component(s) is important in affecting body fat and promoting MT development. What this is remains to be determined.

3-TGF-α/*Lep*$^{+}$*Lep*$^{+}$ Diet-induced Obesity Study.

We enrolled 25 mice in the CHOW-FED group and 51 in the group fed the condensed milk-corn oil-chow (CCC) diet. All mice were homozygous (TGF-α/*Lep*$^{+}$*Lep*$^{+}$) lean female mice. It was previously reported that rats fed this diet either overeat and become obese or restrict their intake and remain at a body weight similar to that of chow-fed rats (26). This resulted in the definition of diet-induced obesity (or OBESITY-PRONE) or diet-resistant (OBESITY-RESISTANT). This diet has also been used in short-term studies to identify strains of mice susceptible toward dietary induced obesity (27). Mice were started on the CCC diet at 10-11 wk of age. Body weights and food intakes were determined weekly and the mice monitored for MT development. Once MT were detected, growth was monitored and mice were killed when tumor size exceeded 20 mm, when excess weight loss occurred or when there were other determining factors. Due to the early development of MT in the OBESITY-PRONE group, this study was terminated at 85 wk of age.

Based on body weight attained at 34 wk of age, we divided the mice fed the CCC diet into three groups. This age was picked because no MT had been detected at this age and the group as a whole weighed significantly more than did the CHOW-FED mice. As shown in Figure 9 (Appendix K), body weights of the 17 heaviest mice termed OBESITY-PRONE were significantly greater than all other groups. Mice with intermediate body weights termed MIDDLE had body weights significantly different from those of the lightest group fed the CCC diet, OBESITY-RESISTANT, but similar body weight as did CHOW-FED mice. The OBESITY-RESISTANT mice also had body weight similar to CHOW-FED mice. During the time from 10-34 wk of age, caloric intakes of the OBESITY-PRONE and CHOW-fed mice were similar and greater than those of the MIDDLE and OBESITY-RESISTANT mice (Figure 10, Appendix L).

To date, 12 mice in the OBESITY-PRONE group are dead due to the presence of MT. The average age of MT detection for the OBESITY-PRONE mice was 47.4 wk of age and this was significantly younger than in any of the other groups. In the MIDDLE group, 12 mice are also dead due to the presence of MT but the age of tumor detection was 58.7 wk of age, while in the OBESITY-RESISTANT group 8 mice are dead with MT and MT detection was at 66.8 wk of age. For CHOW-FED mice, the age of MT detection was 73 wk of age. Finalizing these results will occur when pathology reports are obtained and the remaining mice are killed. Presently, live mice in this protocol range in age from 74-84 wk of age (9/15/00); thus, we anticipate completion of this study shortly after these animals die or are killed.

4- *Lepr*^{db} Mice – Incidence Study

The *Lepr*^{db} mouse strain was successfully cross-bred with the TGF-α mouse strain using a similar strategy as that previously described for the TGF-α/*Lep*^{ob} mice, *i.e.*, nontransgenic female lean (either *Lepr*$^{+}$*Lepr*$^{+}$ or *Lepr*$^{+}$*Lepr*^{db}) mice were mated with TGF-α male mice that were either *Lepr*$^{+}$*Lepr*$^{+}$ or *Lepr*$^{+}$*Lepr*^{db}.

We have completed enrollment of all mice into the Incidence Study for this strain of mice, TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ homozygous lean group (n=40), TGF-α/*Lepr*$^{+}$*Lepr*^{db} heterozygous lean group

(n=39), and in the TGF-α/*Lepr*^{db}*Lepr*^{db} obese group (n=42). Body weight curves for the three groups are shown in Figure 11 (Appendix M). Similar results as those described above for the *Lep*^{ob} mice strain are being obtained. The obese mice weigh significantly more than do the two lean groups when analyzed by ANOVA. When the two lean groups are analyzed separately by Student's "t" test, the *Lepr*$^{+}$*Lepr*^{db} mice weigh significantly more than do the *Lepr*$^{+}$*Lepr*$^{+}$ mice at all 4 week time points from 10 until 74 wk of age. To date, 4 of the 5 dead *Lepr*$^{+}$*Lepr*$^{+}$ mice were killed due to the presence of MT as were 5 of 6 *Lepr*$^{+}$*Lepr*^{db} mice. MT were detected in the homozygous lean mice at 45, 54, 57, and 60 wk of age and at 39, 40, 44, 55, and 57 wk of age for the heterozygous mice. Fifteen obese mice have been killed ranging in age from 12-58 weeks. Live mice in the three groups range in age from 32 to 88 wk of age. We plan on terminating this experiment at 104 wk of age to parallel the protocol for the *Lep*^{ob} Incidence Study, unless there is clear indication that too many mice are dying in order for us to reach that goal.

5- TGF-α/*Lepr*$^{+}$*Lepr*^{db} Weight-Cycling Study

We have been enrolling mice in the weight-cycling protocol as described in the Statement of Work for the third year of the grant. As was done in the *Lep*^{ob} Weight-Cycling Study, we are using heterozygous *Lepr*$^{+}$*Lepr*^{db} mice. Presently, the AD LIBITUM-FED group has 32 mice enrolled (17-57 wk of age), the WEIGHT-CYCLED group 27 mice (10-41 wk of age), and the PAIR-FED group 22 mice (9-24 wk of age) .

6- TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ Diet-Induced Obesity Study

We have enrolled 51 *Lepr*$^{+}$*Lepr*$^{+}$ lean mice in the diet-induced obesity study described in the Statement of Work for the third year of the grant. Feeding of the CCC diet was initiated at 10 wk of age. There are 20 mice enrolled in the CHOW-FED group. These mice currently range in age from 45-64 weeks for CCC-fed and 38-46 weeks for the CHOW-FED group. No MT have been detected in the CHOW-FED group as yet, but 20 of the CCC-fed mice are being monitored for the presence of MT and several have been killed already. We have not divided these mice into OBESITY-PRONE versus OBESITY-RESISTANT groups yet due to the early age of onset of MT in some of the mice fed the CCC diet.

KEY RESEARCH ACCOMPLISHMENTS:

- Established TGF-α /*Lep*^{ob} mouse strain.
- Determined that low level leptin treatment restores fertility to young male obese mice.
- Observed that TGF-α/*Lep*^{ob}*Lep*^{ob} mice have a high mortality rate with mice dying or being euthanized at ages prior to the onset of MT development.
- Found that TGF-α/*Lep*$^{+}$*Lep*^{ob} heterozygous lean mice weigh more than the TGF-α/*Lep*$^{+}$*Lep*$^{+}$ homozygous lean mice, and in addition, the heterozygous mice have an earlier onset of MT and a slightly higher incidence of MT.

- For both *Lep*$^{+}$*Lep*$^{+}$ and *Lep*$^{+}$*Lep*^{ob} mice, higher body and carcass weights were associated with increased risk of MT development. Elevated fat pad weights were also associated with the presence of MT.

- Found that decreased caloric intake, *i.e.*, ~75% of *ad libitum,* was associated with a decreased incidence of MT in TGF-α/*Lep*$^{+}$*Lep*^{ob} mice. This occurred whether caloric restriction was implemented by chronic restriction or by weight-cycling. However, weight-cycling seemed to be more effective in reducing the incidence, as well as increasing the latency for MT development.

- TGF-α/*Lep*$^{+}$*Lep*^{ob} mice that were fed a purified diet based on AIN-93 recommendations for long-term maintenance of rodents were found to have decreased latency for MT development and a slightly increased MT incidence in comparison to mice of the same genotype that were fed commercial rodent chow.

- Mice without the obese *Lep*^{ob} gene, *i.e.*, homozygous *Lep*$^{+}$*Lep*$^{+}$ lean mice, are susceptible to diet-induced obesity when they consume a diet of condensed milk-chow-corn oil. Furthermore, we could identify a group of mice that became obese on the high-fat diet, as well as a group that despite consuming the high-fat diet remained in the body weight range of chow-fed mice.

- OBESITY-PRONE mice have a shortened latency for MT development compared to OBESITY-RESISTANT and CHOW-FED mice. This suggests that a high-fat diet may have differential effect on MT development dependent upon body weight status.

- Established TGF-α/*Lepr*^{db} mouse strain.

- Completed enrollment of mice into TGF-α/*Lepr*^{db} Incidence Study. Initial results demonstrate an effect of genotype on body weight differences between homozygous and heterozygous lean mice similar to that described for the TGF-α/*Lep*^{ob} strain mice.

- Completed enrollment of mice into TGF-α/*Lepr*$^{+}$*Lepr*$^{+}$ diet-induced obesity study. Preliminary results also suggest an early-onset of MT in some of the mice consuming the high-fat diet.

- In collaboration with Drs. Aminah Jatoi, an oncologist at Mayo Clinic, and Dr. Phuong Nguyen, a pathologist at the University of Minnesota, we have found that muscle protein metabolism in MT bearing mice is affected prior to any detrimental effects on body weight.

- In collaboration with Dr. Gina Pighetti from Pennsylvania State University we are investigating the potential role of leptin and leptin receptors in the development of mammary tumors. We have sent her samples from MT and adipose tissue and normal mammary epithelial tissue for analysis of the presence of RNA and protein for these compounds.

REPORTABLE OUTCOMES:

Presentations

Invited speaker, "The Effect of Obesity on the Development of Mammary Tumors," Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN October 1999

Invited speaker, "Body Weight and the Development of Breast Cancer," Lyle Eagles, Lyle, MN April 1999.

Poster presentation, "TGF-α/*Lep*$^{+}$*Lep*^{ob} lean mice weigh more and have higher mammary tumor (MT) incidence than do TGF-α/*Lep*$^{+}$*Lep*$^{+}$ lean mice" 91st American Association for Cancer Research meeting, San Francisco, CA, April 2000. *Proc. Am. Assoc. Cancer Res.* **41,** 83-84 (2000).

Poster presentation, "Weight gain does not preclude activation of the ubiquitin-proteasome system in skeletal muscle of tumor-bearing mice". 91st American Association for Cancer Research meeting, San Francisco, CA, April 2000. *Proc. Am. Assoc. Cancer Res.* **41,** 200 (2000).

Poster presentation, "Weight-cycling of TGF-α/*Lep*$^{+}$*Lep*^{ob} female mice: effects on oncogene-induced mammary tumors (MTs), body weight, and food intake". Experimental Biology meeting San Diego, CA, April 2000.

Invited talk, "Elevated body and carcass weights are associated with a higher incidence of proto-oncogene induced mammary tumors in MMTV-TGF-α mice". DOD Era of Hope meeting, Atlanta, GA, June 2000.

Poster presentation, "Increased incidence of mammary tumors in MMTV-TGF-α/*Lep*$^{+}$*Lep*$^{+}$ obesity-prone mice in comparison to MMTV-TGF-α/*Lep*$^{+}$*Lep*$^{+}$ obesity-resistant mice". DOD Era of Hope meeting, Atlanta, GA, June 2000.

Poster presentation, "Elevated body and carcass weights are associated with a higher incidence of proto-oncogene induced mammary tumors in MMTV-TGF-α mice". DOD Era of Hope meeting, Atlanta, GA, June 2000.

Manuscripts

Jatoi, A., M.P. Cleary, C.-M. Tee, and P.L. Nguyen. Weight gain does not preclude activation of the ubiquitin-proteasome system in skeletal muscle of tumor-bearing mice. Annals of Nutrition and Metabolism (accepted pending revisions). (Appendix N)

Cleary, M.P., H.M. Bergstrom, T.L. Dodge, S.C. Getzin, M.K. Jacobson, and F.C. Phillips. Restoration of fertility in young obese (*Lep*^{ob}*Lep*^{ob}) male mice with low dose recombinant mouse leptin treatment. International Journal of Obesity (in press). (Appendix O)

CONCLUSIONS:

This research project has gone very well although maybe not as quickly, as we would have liked to see more conclusive results at this point. We have met our goals for the project and are now seeing exciting results. Completion of the three ongoing protocols with the TGF-*Lep*^{ob} strain mice in the next few months will allow us to be definitive about some of our conclusions. Interesting findings to date include the heterozygous effect of the presence of both the *Lep*^{ob} and *Lepr*^{db} genes in lean mice results in a higher body weight compared to homozygous lean mice. Furthermore, this increase in body weight was associated with an increased risk of MT development in the TGF-α/*Lep*$^{+}$*Lep*^{ob} mice compared to the TGF-α/*Lep*$^{+}$*Lep*$^{+}$ mice, as well as an earlier age of MT onset and death. It remains to be determined if body fat in these mice is also higher, although fat pad weights were elevated. Further analysis of tissues and blood samples should shed further insights into interpreting these findings. The observation that some homozygous lean mice can become obese when challenged with a high-fat diet is also of interest. This is the first report that mice respond to this diet in a similar manner as do several strains of rats. Although it is not clear yet whether the increase in body weight is associated with an increased MT incidence, it definitely appears that the increase in body weight is associated with a shortened latency for MT development and shortened life expectancy. This model has provided the opportunity to compare mice of similar diets with different body weights, as well as mice with similar body weights but different diets. We hope that this will help to clarify the role of dietary fat level in MT development. The results from the weight-cycling study are also very interesting, indicating that caloric restriction and diet composition independent of body weight can impact MT latency and incidence. Although it was disappointing initially to have a high and early mortality rate in the genetically obese mice, the results of the dietary studies potentially have a much greater impact for application to human studies than would results from the genetically obese mice.

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APPENDIX A- ORIGINAL STATEMENT OF WORK

III.B.2.e. Statement of Work

Months 1-2. Order breeding animals, racks and initial supplies. Set up assays for trans-oncogene and *ob* determinations.

Months 3-5. Initiate first set of breedings and determine genotype of offspring.

Months 5-6. Initiate matings of double heterozygous mice. Enroll female homozygous lean (+/+) and obese (*ob/ob*) mice in breast cancer incidence study. Test for genotypes and take blood samples. Weigh experimental mice.

Months 6-12. Continue double heterozygous matings and start homozygous (**TT**) trans-oncogene matings. Continue to enroll female homozygous lean (+/+) and obese (*ob/ob*) mice from both mating groups in the incidence study. Test for genotypes and take blood samples. Weigh mice and examine for tumors weekly. Monitor tumor growth.

Months 13-18. Continue to monitor mice enrolled in the incidence study. Collect samples from ethanized mice. When 25 mice enrolled per lean and obese groups, inititate weight-cycling study.
Monitor those mice, take blood samples and prepare and feed special diets.

Months 15-18. Order *db* mice and inititate that breeding colony. Enroll *ob* mice in weight-cycling study and record weekly weights and food intakes and monitor tumor incidenc and growth in these mice. Prepare and feed special diets.

Months 18-21. Initiate matings of double heterozygous trans-oncogene *db* mice. Enroll female homozygous lean (+/+) and obese (*db/db*) mice in breast cancer incidence study. Test for genotypes and take blood samples. Weigh and monitor experimental mice and continue monitoring food intakes of weight-cycled mice. Continue diet preparations. Kill mice from the incidence study as they reach 16 months of age. Do body compositions on mice from incidence study. Enroll *ob* strain mice in diet-induced obesity study and monitor.

Montha 22-28. Continue heterozygous matings and start homozygous (**TT**) trans-oncogene matings of *db* mice colony. Continue to enroll *db* strain in incidence study and weigh mice, examine for tumors and monitor tumor growth of all experimenatal mice. Kill weight-cycled mice as they reach 16 months of age. Do cellularity and body compostion determinations. Kill mice from db incidence study and enroll additional animals in weight-cycling protocol.

Months 24-36. Record food intakes, body weight and monitor for tumors and tumor growth. Kill remaing weight-cycled mice and kill diet-induced obese *ob* mice as they reach 16 months of age. Perform cellularity measurements and body compostition analysis. Kill *db* mice from incidence study.

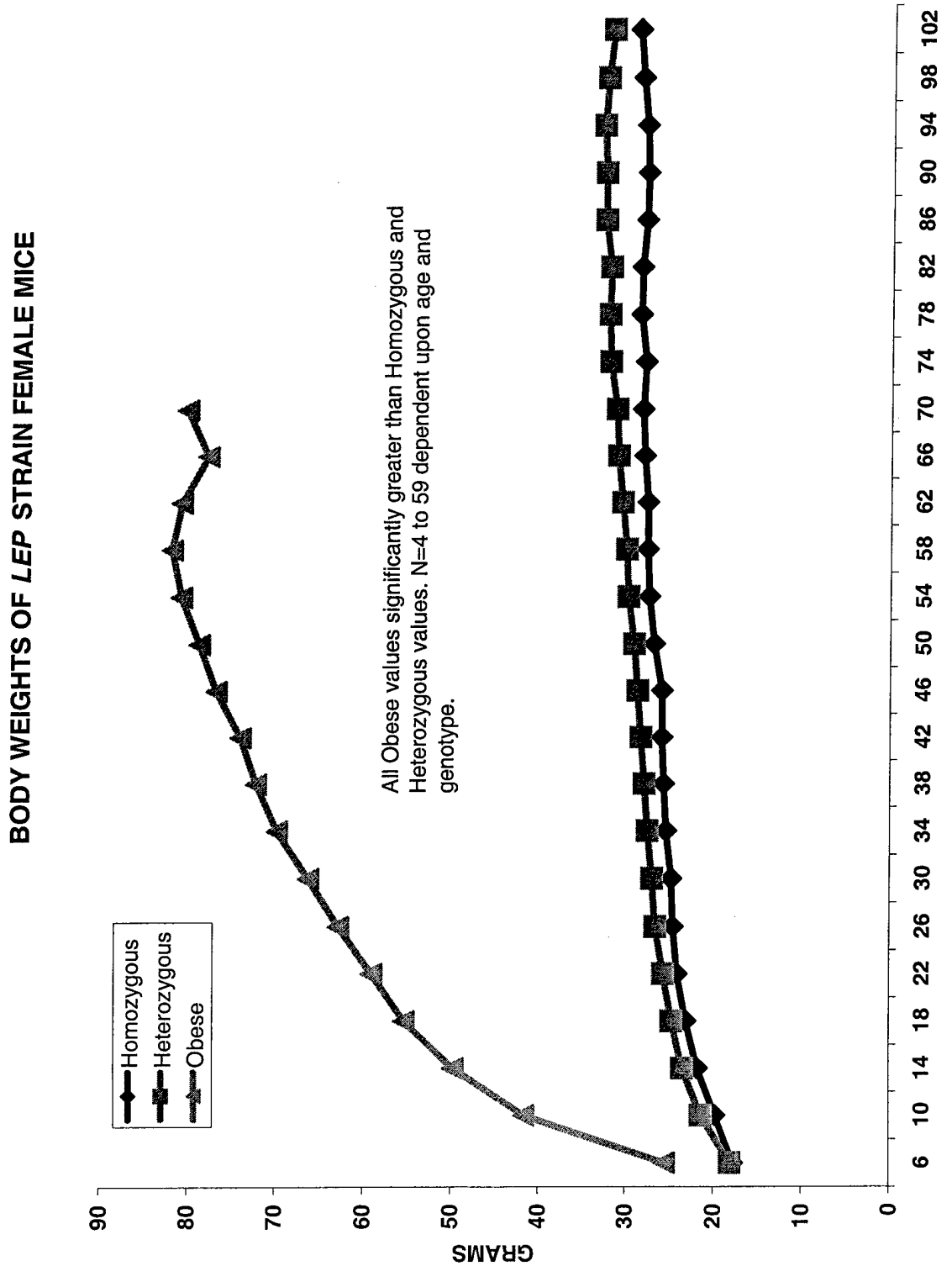


FIGURE 1

APPENDIX B

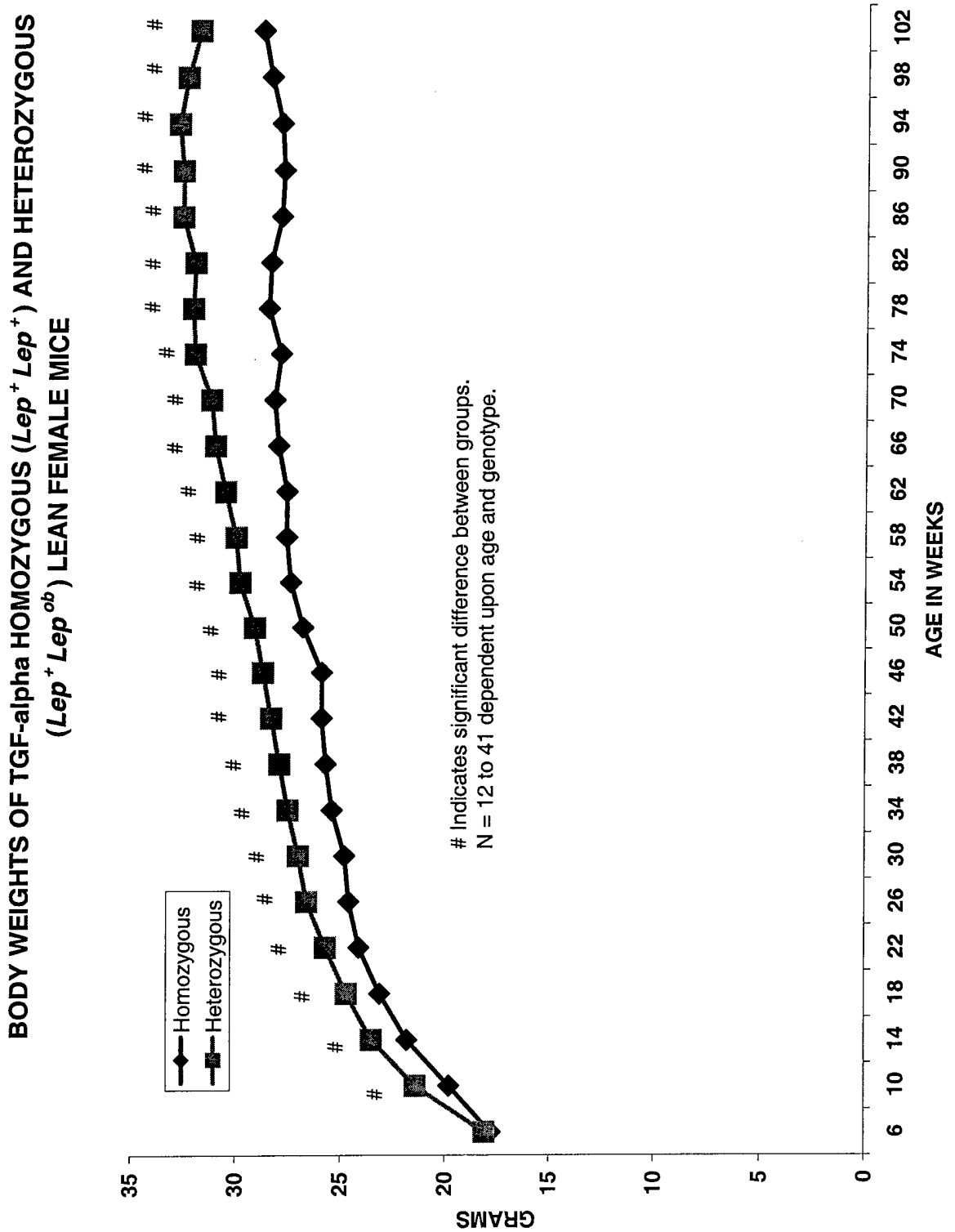


FIGURE 2

APPENDIX C

SURVIVAL CURVES FOR TGF-α/*Lep*^{ob} FEMALE MICE

Curves significantly different at p <0.0001
Median survival-
Obese 54 weeks, HET-LEAN 89 weeks, HOM-LEAN 104 weeks

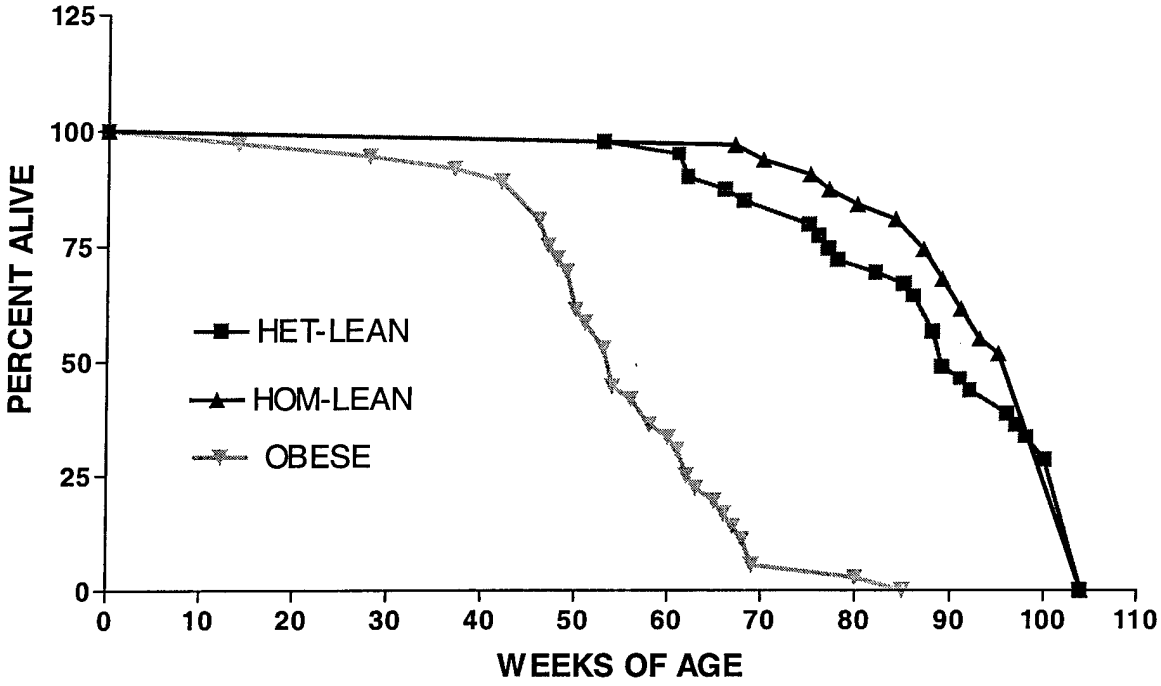


TABLE 1

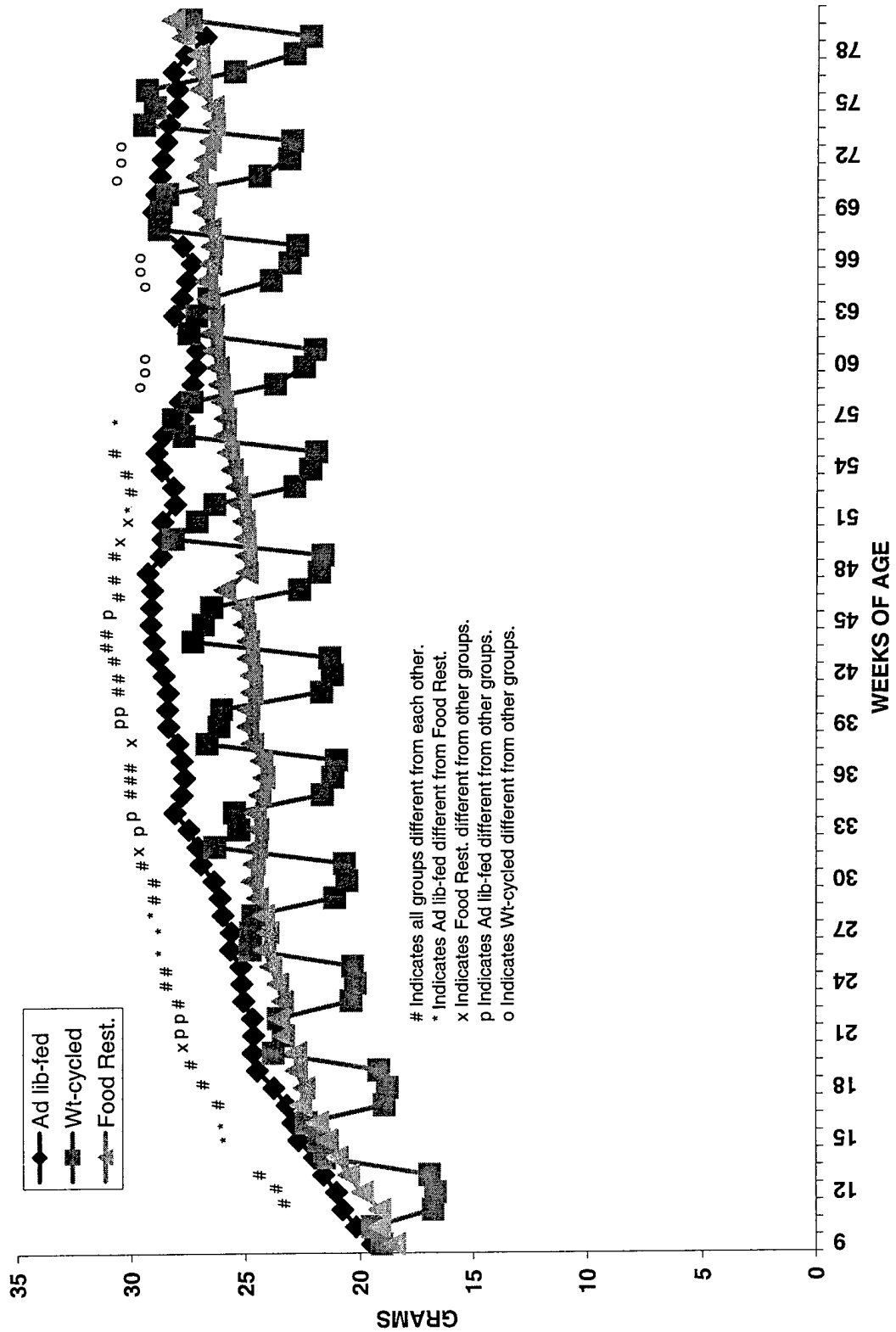
Final body weight, carcass weight, combined fat pad weights and tumor burden for homozygous and heterozygous lean female TGF-α/*Lep* mice

	BODY WEIGHT (GRAMS)	CARCASS WEIGHT (GRAMS)	COMBINED FAT PAD WEIGHT (GRAMS	NUMBER OF MAMMARY TUMORS	TUMOR BURDEN (GRAMS)
HOMOZYGOUS LEAN NO MT (n=18)	26.78±1.02$	23.26±0.80$	0.523±0.079$		
HOMOZYGOUS LEAN WITH MT (n=17)	32.77±1.22#	28.21±0.88#	0.628±0.0.080#	4.25±0.60	1.891±0.422
HETEROZYGOUS LEAN NO MT (n=13)	32.62±1.10$	28.09±0.97$	0.714±0.125$		
HETEROZYGOUS LEAN WITH MT (n=26)	35.90±1.13#	30.90±0.91#	1.023±0.158#	2.5±0.58	1.958±0.52

Values are means ± sem.

$ Indicates significant effect of genotype by 2-way ANOVA.

# Indicates significant effect of MT by 2-way ANOVA.


BODY WEIGHTS OF MICE FROM TGF-alpha/Lep+ Lepob WEIGHT-CYCLING STUDY
Ad lib-fed
Wt-cycled
Food Rest.
GRAMS
WEEKS OF AGE
# Indicates all groups different from each other.
* Indicates Ad lib-fed different from Food Rest.
x Indicates Food Rest. different from other groups.
p Indicates Ad lib-fed different from other groups.
o Indicates Wt-cycled different from other groups.

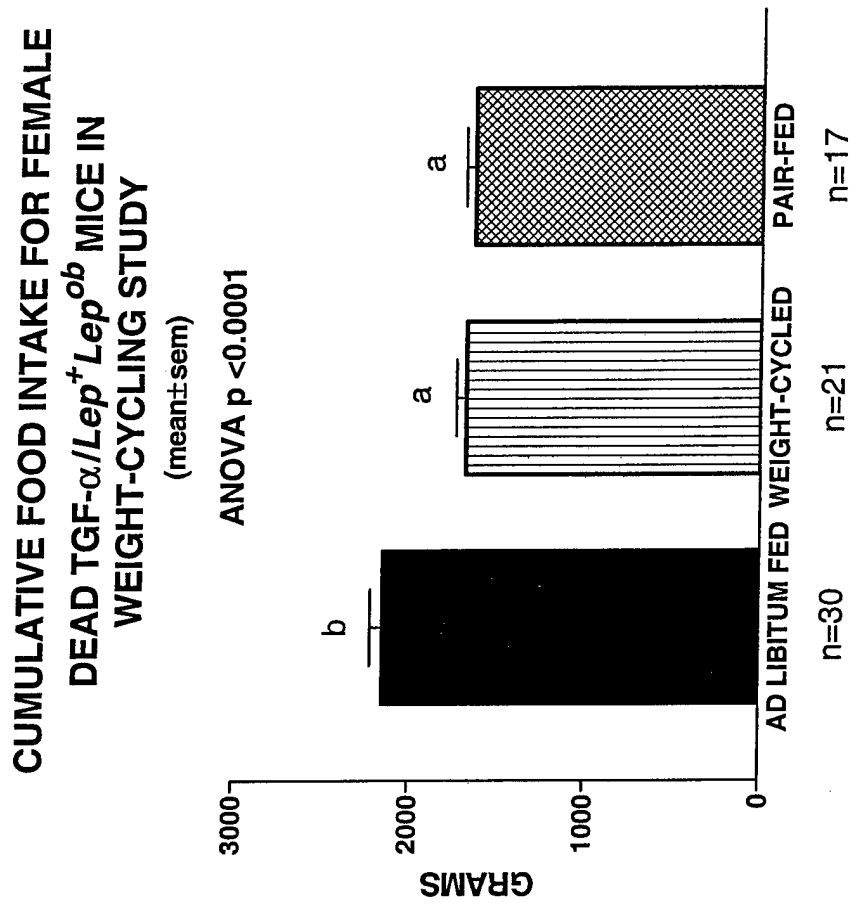

CUMULATIVE FOOD INTAKE FOR FEMALE
DEAD TGF-α/Lep+Lepob MICE IN
WEIGHT-CYCLING STUDY
(mean±sem)
ANOVA p <0.0001
GRAMS
3000
2000
1000
0
b
a
a
AD LIBITUM FED
WEIGHT-CYCLED
PAIR-FED
n=30
n=21
n=17

FIGURE 5

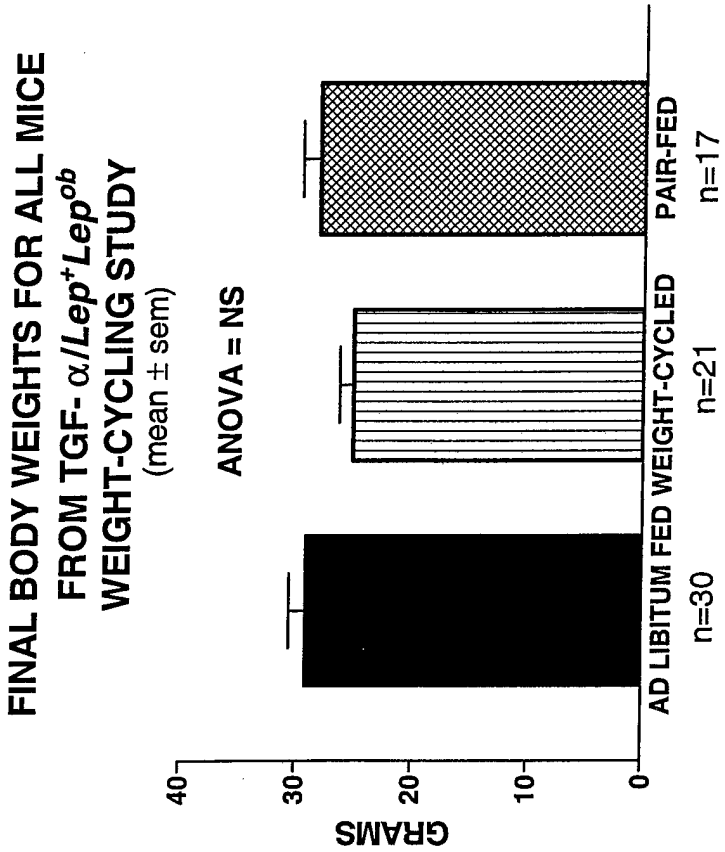

FINAL BODY WEIGHTS FOR ALL MICE
FROM TGF- α/Lep+Lepob
WEIGHT-CYCLING STUDY
(mean ± sem)
ANOVA = NS
GRAMS
40
30
20
10
0
AD LIBITUM FED
n=30
WEIGHT-CYCLED
n=21
PAIR-FED
n=17

FIGURE 6

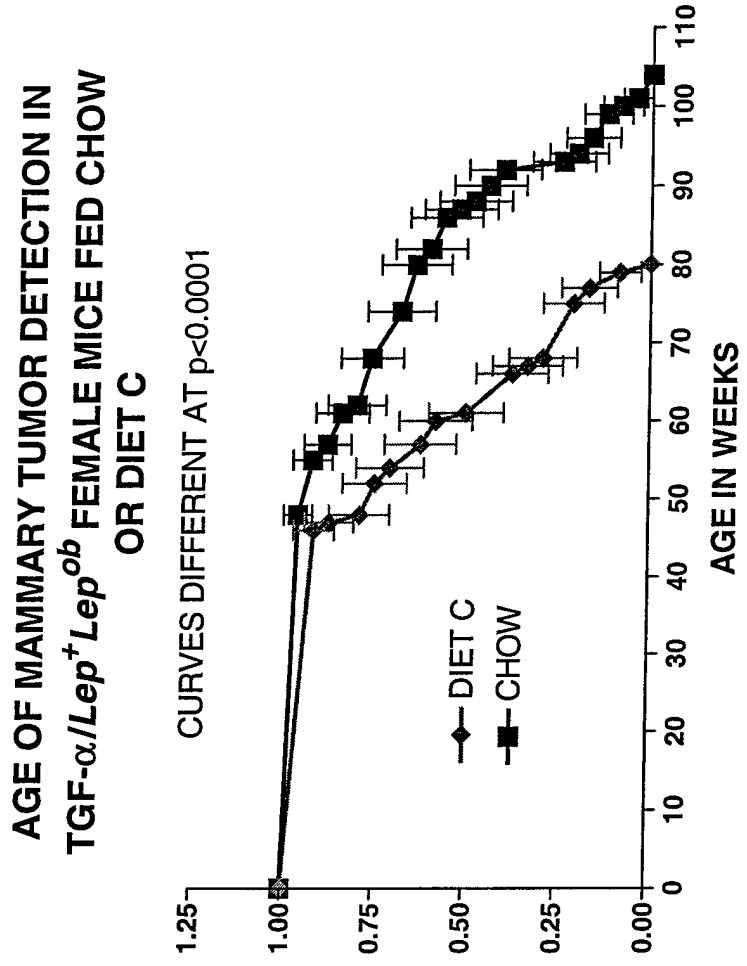


FIGURE 7

APPENDIX I

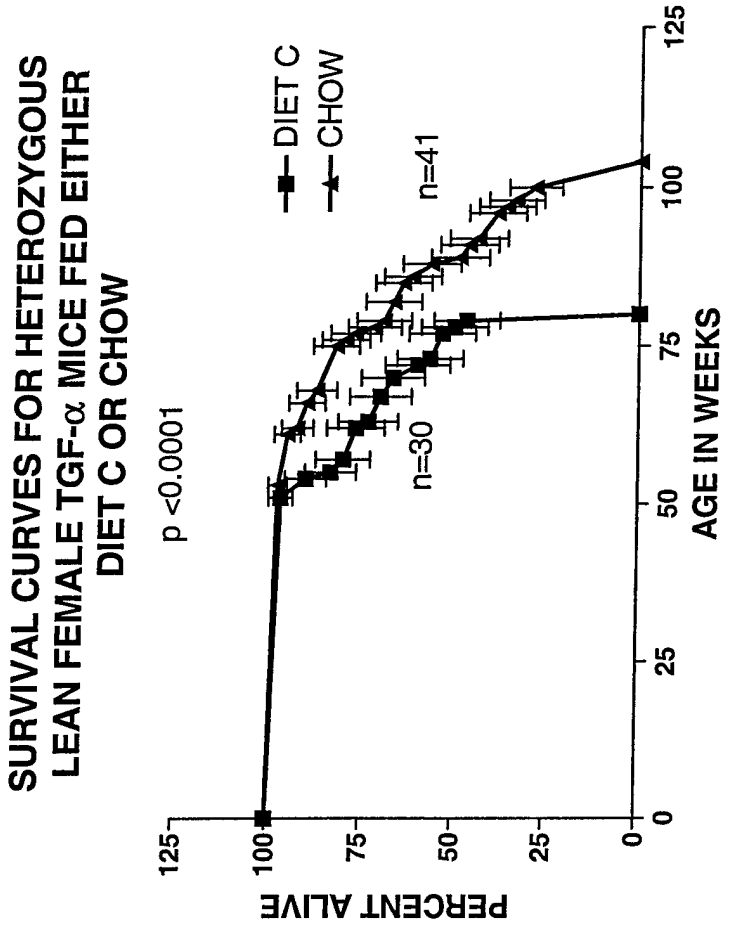

SURVIVAL CURVES FOR HETEROZYGOUS
LEAN FEMALE TGF-α MICE FED EITHER
DIET C OR CHOW
p <0.0001
DIET C
CHOW
n=41
n=30
PERCENT ALIVE
AGE IN WEEKS
125
100
75
50
25
0
0
25
50
75
100
125

FIGURE 8

FIGURE 9

BODY WEIGHTS AT 34 WEEKS OF AGE FOR TGF-α/*Lep*$^{+}$*Lep*$^{+}$ FEMALE MICE FED CMD OR CHOW (mean ± sem)

ANOVA p = 0.0001, columns with different superscripts are significantly different

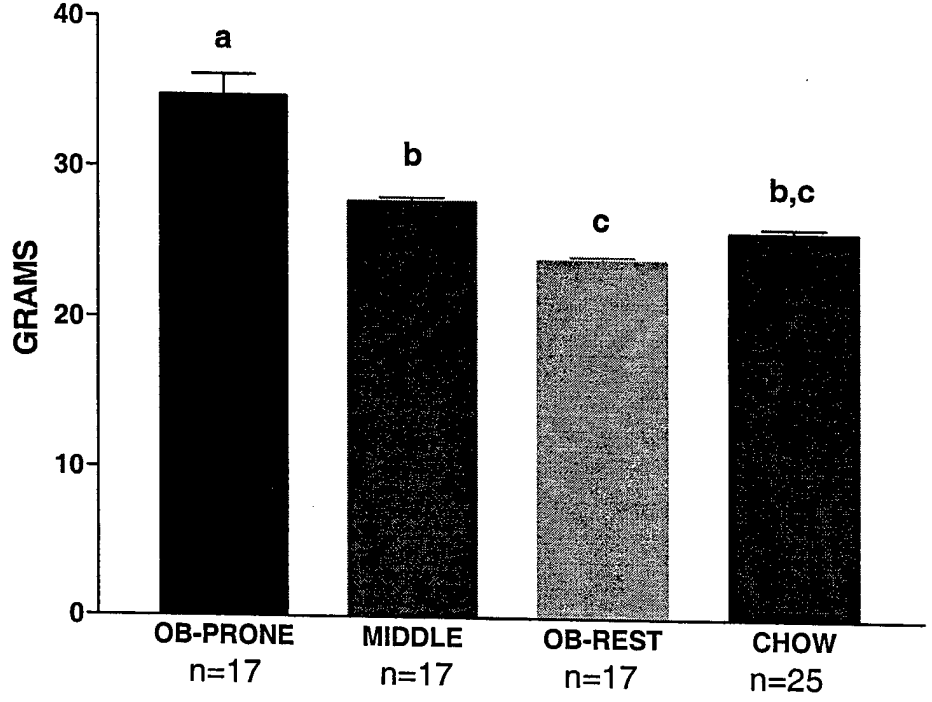
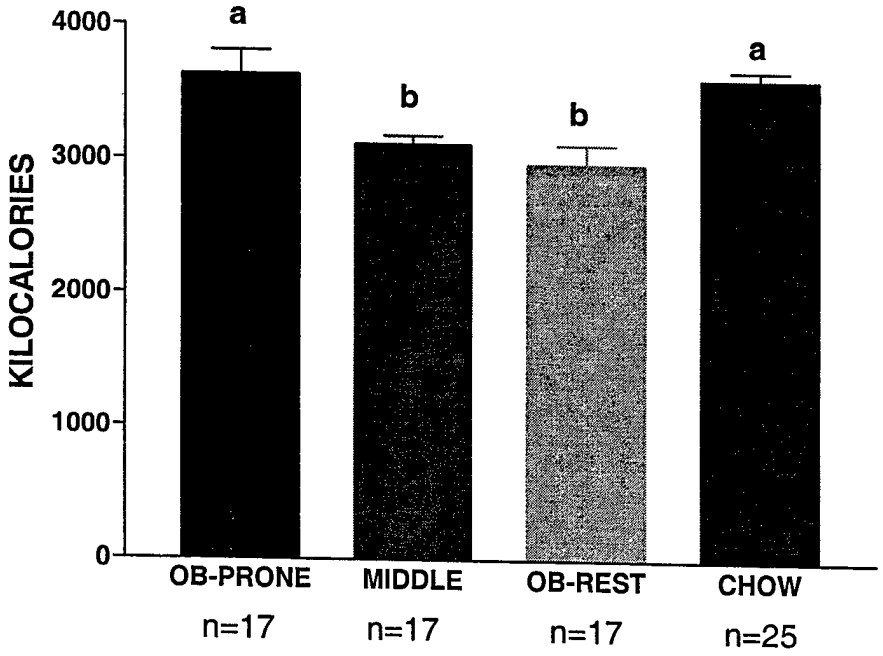


FIGURE 10

CALORIC INTAKE FROM 10-34 WEEKS OF AGE OF TGF-α/*Lep*$^{+}$*Lep*$^{+}$ FEMALE MICE FED CMD OR CHOW

(mean ± sem)

ANOVA p = 0.0002, columns with different superscripts are significantly different



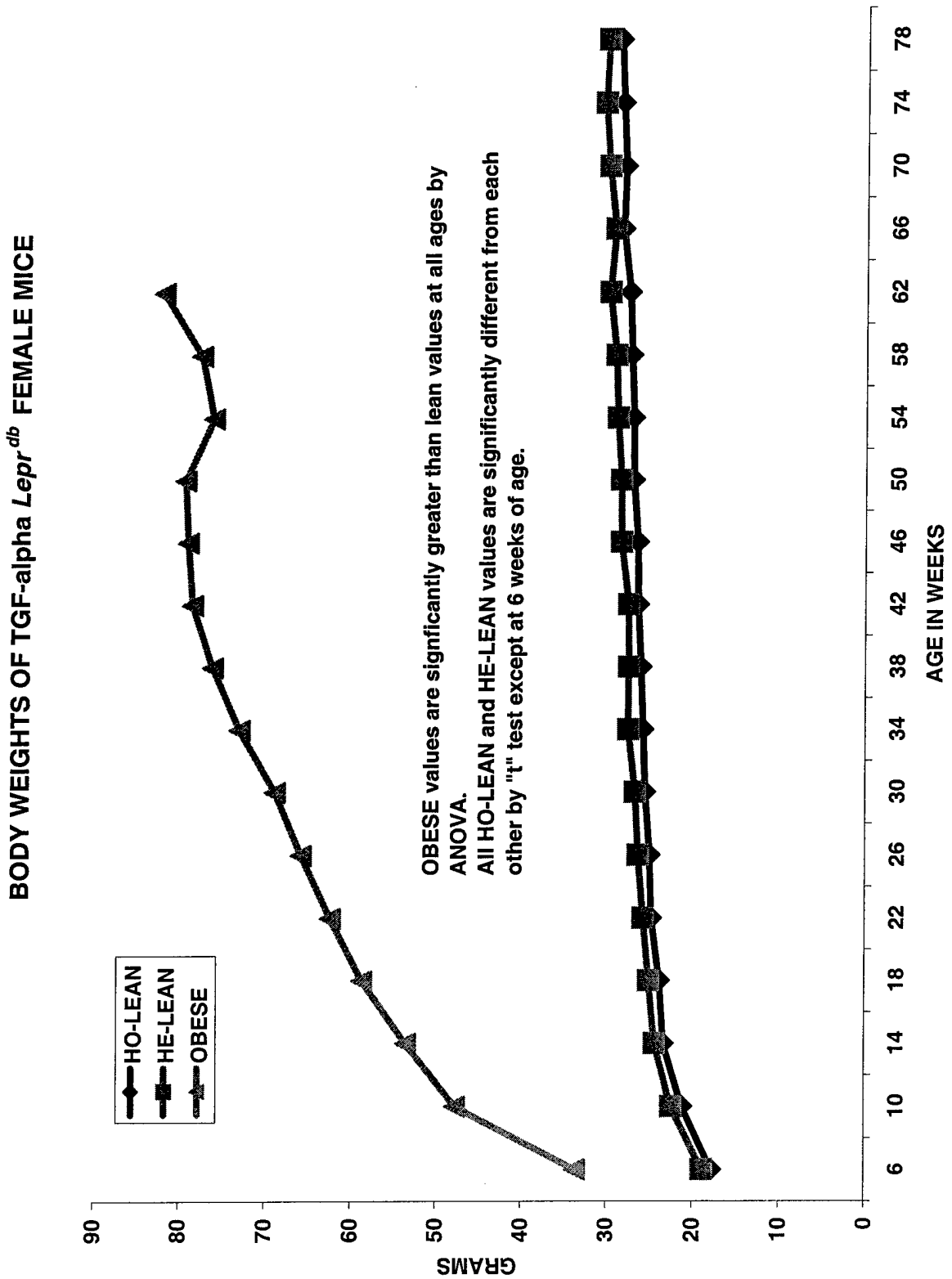


FIGURE 11

APPENDIX M

APPENDIX N

Annals of Nutrition and Metabolism
(accepted pending revision)

Weight Gain Does Not Preclude Increased Ubiquitin Conjugation in Skeletal Muscle: An Exploratory Study in Tumor-Bearing Mice

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Running Title: Ubiquitin conjugates in weight-gaining, tumor-bearing mice

Key words: cancer, cachexia, wasting, muscle, ubiquitin

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ABSTRACT

Background and Hypothesis: At least 13 studies have shown that the ubiquitin-proteasome system mediates muscle wasting in weight-losing cancer subjects. We hypothesized that with cancer, the ubiquitin-proteasome system is activated, regardless of weight loss.

Methods: We utilized hybrid mice obtained by crossing Mouse Mammary Tumor Virus -Transforming Growth Factor-α (TGF-α) mice with the *Lep*^{ob} strain. Five hybrid MMTV-TGF-α heterozygous *Lep*$^{+}$*Lep*^{ob} female mice with mammary tumors were used; 4 non-transgenic heterozygous *Lep*$^{+}$*Lep*^{ob} female mice served as controls. Ubiquitin conjugates were quantitated from hamstring and paraspinal muscles by Western blotting. Myocyte apoptosis was determined by a modified tunel assay.

Results: All mice gained weight, even after tumor development. Higher concentrations of muscle ubiquitin conjugates were seen in the 5 tumor-bearing, TGF-α transgenic mice compared to the 4 non-tumor-bearing mice: median [range] in arbitrary densitometric units: 0.67 [0.22-4.59] versus 0.18 [0.08-0.44] in hamstring muscle and 0.56 [0.23-20.15] versus 0.18 [0.08-0.25] in paraspinal muscle ($p=0.04$ and $p=0.04$, respectively; Mann Whitney U test). Apoptosis was not seen in any muscle sample studied.

Conclusions: We observed increased ubiquitin conjugates in the skeletal muscle of tumor-bearing, weight-gaining mice. Thus, a major mechanism of cancer-associated muscle wasting appears to be activated in the absence of weight loss.

INTRODUCTION

Over 13 studies have demonstrated that the ubiquitin-proteasome system mediates muscle breakdown in cancer wasting [1-13]. The ubiquitin-proteasome system is an ATP-dependent pathway that leads to breakdown of senescent or damaged proteins [14]. In the presence of cancer, this system is the best-described and most active mechanism by which muscle wasting occurs. It appears to be responsible for over 80% of cancer-associated muscle proteolysis [2]. In effect, the ubiquitin-proteasome system causes the debilitating and excessive loss of lean tissue that is the hallmark of cancer-associated wasting [15].

Up until now, all previous investigations of muscle wasting in cancer have focused on tumor-bearing subjects who have lost weight. To our knowledge, no prior studies have examined tumor-bearing subjects who have maintained or gained weight. However, recent data suggest that some of the metabolic changes seen in cancer-associated wasting occur prior to weight loss [16]. We hypothesized that weight loss may in fact be a late phenomenon in cancer-associated wasting and that the presence of the tumor itself activates the ubiquitin-proteasome pathway, thus leading to this preferential wasting of lean tissue even before weight loss becomes manifest.

To test our hypothesis, we undertook an exploratory investigation of ubiquitinated muscle proteins in a transgenic, tumor-bearing mouse model. The present study represents an investigation within a much larger effort aimed at determining the interrelation of obesity and/or body weight with respect to

mammary tumorigenesis. Hybrid mice were obtained by crossing the MMTV-TGF-α strain [16] with the genetically obese *Lep*^{ob} strain [17]. Female heterozygous *Lep*$^{+}$*Lep*^{ob} mice, which weighed significantly more than the homozygous *Lep*$^{+}$*Lep*$^{+}$ mice [18], were used in this study to explore activation of the ubiquitin-proteasome system in skeletal muscle in the absence of weight loss.

SUBJECTS AND METHODS

Animals: A total of 9 female mice were studied. Mice were obtained by crossing the MMTV-TGF-α strain originally described by Matsui et al [16] with *Lep*^{ob} mice. Female offspring were genotyped to determine the presence of the TGF-α gene and to determine the *Lep* genotype. Only heterozygous *Lep* $^{+}$*Lep*^{ob} mice were used in the present study. Five transgenic (MMTV-TGF-α) mice that developed mammary tumors were used; four *Lep*$^{+}$*Lep*^{ob} mice that did not overexpress TGF-α and did not develop mammary tumors served as control subjects.

With approval by the University of Minnesota Institutional Animal Care and Use Committee, the mice were maintained in pairs in a temperature (21°C) and humidity (50%) controlled room with a 12 hour light/dark cycle at the animal facility at the Hormel Institute, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. All mice were fed *ad libitum* with standard rodent food (Purina Rodent Chow® 5001) and were allowed free access to water. All 5 transgenic mice were weighed weekly over a period of one year and were palpated weekly to check for presence of tumor. The 5 mice who developed tumors continued to be weighed after tumor development. The other 4 mice were weighed once a month. Tumor-bearing mice were sacrificed when the weight of the tumor(s) exceeded 10% of total body weight or when animal caregivers deemed that the animals' quality of life had deteriorated

to an unacceptable level. Control subjects were sacrificed upon reaching 12-14 months of age.

At the time of sacrifice by exsanguination, mouse paraspinal and hamstring muscles were collected and snap-frozen in liquid nitrogen. All tumors were collected and examined for histologic confirmation of malignancy. In addition, kidneys, lungs, liver, heart, spleen and ovaries were collected in all tumor-bearing animals and were examined microscopically for evidence of metastatic spread of tumor.

Extraction of muscle proteins: Frozen muscle samples were homogenized in protein extraction buffer (50 mM Tris base [pH 7.6], 1 μg/ml Aprotinin, 1 μg/ml Antipain, 1 μg/ml Leupeptin, and 1 μg/ml Pepstatin A [Sigma Chemical Company, St. Louis, MO]). Homogenates were centrifuged and supernatants stored at -80°C. Protein concentrations were determined with the BCA Protein Assay Kit (Pierce; Rockford, IL).

Western blot: Supernatants containing 2 μg of protein per sample per lane were loaded onto 16.5% Tris Tricine acrylamide gradient gels (BioRad Laboratories, Hercules, CA) and electrophoresed. Gels were then electro-blotted onto 0.2-micron nitrocellulose membrane (BioRad Laboratories, Hercules, CA). Unbound sites on the membrane were then blocked overnight at 4°C in 3% Bovine Serum Albumin, after which the membrane was incubated with a primary mouse anti-ubiquitin monoclonal antibody that recognizes both free and conjugated ubiquitin (Chemicon International Inc., Temecula, CA), at 40-100 μg/ml. The membrane was incubated in ECL Western Blotting Detection

reagents (Amersham Pharmacia Biotech, Piscataway, NJ) before being exposed for 1 minute to Fuji Medical X-ray Film (Stamford, CT). Quantitation of ubiquitin conjugates was determined using a BioRad GS-700 imaging densitometer. All readings were normalized to extracted protein concentrations.

Evaluation of myocyte apoptosis: As an exploratory component of this study, muscle samples were also examined for evidence of apoptosis using a modified tunel assay (ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit; Intergen, Purchase, NY). Following de-paraffinization and rehydration, sections of formalin-fixed, paraffin-embedded muscle samples were treated with proteinase K at room temperature at 40 μg/ml (Roche Molecular Biochemicals; Indianapolis, IN) and quenched in 3% hydrogen peroxide before labeling with digoxigenin-labeled nucleotides using the enzyme terminal deoxynucleotidyl transferase (TdT) for 1 hour at 37^0C. The incorporated nucleotides were then detected with a biotinylated anti-digoxigenin antibody followed by a strepavidin-horseradish peroxidase conjugate and diaminobenzidine chromogen detection reagent.

Sections of a human lymph node with hyperplastic germinal centers served as a positive control for apoptosis. In addition, to confirm that this procedure is capable of detecting DNA fragments, a consecutive but separate section of each muscle sample was subjected to the identical procedure but with a DNase treatment step between proteinase K digestion and quenching. Sections were examined under the microscope for evidence of apoptosis, which consisted of brown nuclear staining in myocytes with pyknotic nuclei.

Data analysis: Data are presented descriptively or as median values with ranges. The Mann Whitney U test was used to make direct comparisons between muscle in tumor-bearing and non-tumor-bearing animals. A p-value of < 0.05 was considered statistically significant. Results were analyzed with SyStat 5.2.1 for MacIntosh (SPSS, Chicago, Illinois, USA).

RESULTS

All 9 mice demonstrated a progressive gain in weight throughout the study, even after tumor development (Figure 1). Tumors had developed between 48 and 68 weeks of age (median: 61 weeks) in the transgenic mice; these tumor-bearing mice were sacrificed at 9-15 weeks after tumor detection. At the time of death, tumor-bearing mice ranged in age from 62 to 76 weeks (median: 75 weeks); non-tumor-bearing animals were 56 - 58 weeks in age (median: 58 weeks). At the time of death, tumor-bearing mice weighed more than control mice: median total body weight [range], 39.4 grams [33.2 - 42.8] versus 27.4 grams [26.6 - 29.0], $p = 0.02$ (Mann-Whitney U test). However, there was no statistically significant difference between the 2 groups with respect to carcass weights, defined as the difference between total body weight and the sum of tumor and organ weights (heart, lungs, liver, kidneys, and spleen) [18]: median carcass weight [range]: 33.6 grams [26.0 - 36.9] for the tumor-bearing mice versus 25.2 grams [24.4 - 26.8] for the non-tumor-bearing mice ($p=0.10$) (Mann-Whitney U test).

All 5 tumor-bearing animals had their tumor's malignant histology confirmed by microscopic examination by a pathologist; none of the 4 non-transgenic mice had gross evidence of tumor at death. Among the tumor-bearing animals, all tumors had the histologic characteristics of mammary adenocarcinoma. When all other organs were examined microscopically, only one animal had evidence of metastatic disease. This animal had a malignant

cellular infiltrate that involved the renal medullae. All mice were genotyped to confirm whether or not they carried the MMTV-TGF-α gene and to confirm their status with respect to the *Lep* genotype.

Immunoblotting with monoclonal anti-ubiquitin antibody demonstrated higher concentrations of muscle ubiquitin conjugates in the 5 tumor-bearing mice compared to the 4 non-tumor-bearing mice (Figure 2): median [range], in densitometric units: 0.67 [0.22-4.59] versus 0.18 [0.08-0.44] in hamstring muscle and 0.56 [0.23-20.15] versus 0.18 [0.08-0.25] in paraspinal muscle, ($p=0.04$ and $p=0.04$, respectively; Mann-Whitney U test). (Figures 3 and 4, respectively.)

The modified tunel assay was performed in 4 of 5 hamstring muscle samples and 5 of 5 paraspinal muscle samples in tumor-bearing mice and in 3 of 4 hamstring muscle samples and 4 of 4 paraspinal muscle samples in non-tumor-bearing mice. Not all muscles were evaluated because of lack of adequate tissue. No evidence of apoptosis was seen in any muscle sample. Sections of the hyperplastic lymph node and DNase-treated sections did, however, show appropriate positive staining.

DISCUSSION

To our knowledge, this exploratory study is the first to demonstrate an increase in ubiquitin conjugates in muscle of tumor-bearing subjects in the absence of weight loss. Although our study did not specifically examine muscle proteolysis, our findings suggest that the presence of cancer itself activates the ubiquitin-proteasome system, a pathway well-recognized for its role in muscle breakdown. Since the ubiquitin-proteasome system accounts for over 80% of muscle wasting associated with cancer [2], evidence of early activation of this system might carry major clinical importance and suggests that occult wasting of lean tissue may precede loss of weight.

The prognostic significance of weight loss in cancer is well-known [20], and the adverse impact of wasting of lean tissue has also been well characterized. Cohn and others have demonstrated that weight loss in cancer patients represents an excessive and disproportionate loss of muscle tissue in contrast to the tissue loss observed in other body compartments such as fat [21]. It is this loss of muscle tissue that leads to much of the morbidity associated with cancer, including decline in performance status and predisposition to infection [15]. Hence, early activation of the ubiquitin-proteasome system may adversely impact the tumor-bearing host even before weight loss becomes manifest.

As mentioned earlier, we did not specifically examine muscle proteolysis in this study. Our results are only suggestive of early occult wasting of muscle in tumor-bearing animals. It is possible that following activation, the ubiquitin-

proteasome system degrades other proteins besides muscle proteins. Alternatively, it is possible that conjugation of muscle proteins occurs but that but that ultimately these proteins are not degraded. Our early observations underscore the importance of further investigating mechanisms of muscle wasting even in the presence of early-stage malignancies and even in the absence of weight loss.

As an exploratory component of our study, we had looked for evidence of apoptosis in muscle samples. Apoptosis of myocytes occurs in other clinical settings involving muscle wasting [22,23]; but, to our knowledge, prior studies have not examined myocyte apoptosis in the setting of cancer-associated wasting. While our preliminary data suggest that apoptosis of myocytes does not occur in cancer-associated wasting, other possibilities besides absence of apoptosis in this setting may account for these negative results. These possibilities include a lack of adequate power in this exploratory component of the study or less than optimal timing as to when we had looked for apoptosis. With regard to the latter possibility, myocyte apoptosis may in fact occur with cancer-associated wasting but only as a late-stage phenomenon that accompanies weight loss.

In summary, our study demonstrates that increased ubiquitin conjugation occurs in skeletal muscle of tumor-bearing animals in the absence of weight loss. Our results suggest that it may be appropriate to investigate strategies to halt tissue wasting prior to weight loss.

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FIGURE LEGENDS:

Figure 1: Both tumor-bearing mice (thin line with triangles) and non-tumor-bearing mice (thicker line) demonstrated a progressive increase in weight during the 12 months preceding death, which continued even after tumor development in the transgenic mice.

Figure 2: Immunoblot of hamstring (lanes 2 and 5) and paraspinal muscle samples (lanes 3, 4, 6, and 7) from tumor-bearing mice (lanes 2-4) and non-tumor-bearing mice (lanes 5-7). 1 μg of protein was loaded onto each lane. Lane 1 is the positive control showing free ubiquitin at 8.5 kD. Ubiquitin conjugates are seen at higher molecular weights.

Figure 3: Ubiquitin conjugates in mouse hamstring muscle were significantly higher in tumor-bearing animals than in non-tumor-bearing animals (median [range] in arbitrary densitometric units: 0.67 [0.22-4.59] versus 0.18 [0.08-0.44]; p=0.04; Mann Whitney U test).

Figure 4: Ubiquitin conjugates in mouse paraspinal muscle were significantly higher in tumor-bearing animals than in non-tumor-bearing animals (mean [range] in arbitrary densitometric units: 0.56 [0.23-20.15] versus 0.18 [0.08-0.25]; p=0.04; Mann Whitney U test).

FIGURE 1

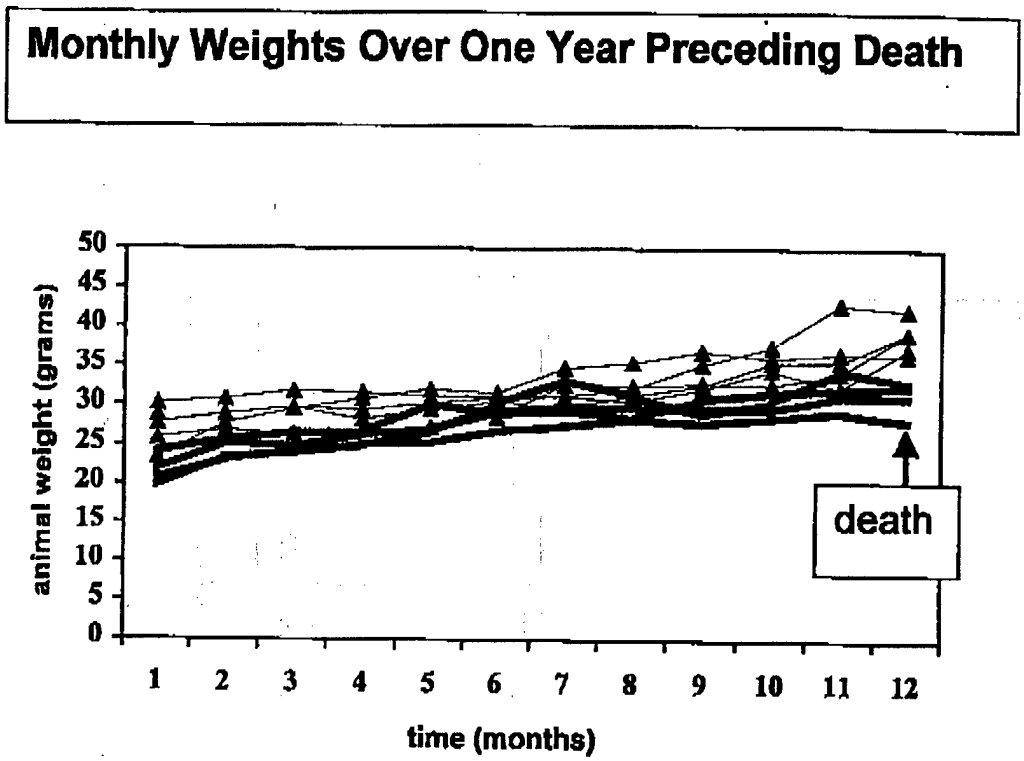

Monthly Weights Over One Year Preceding Death
animal weight (grams)
50
45
40
35
30
25
20
15
10
5
0
1
2
3
4
5
6
7
8
9
10
11
12
time (months)
death

FIGURE 2

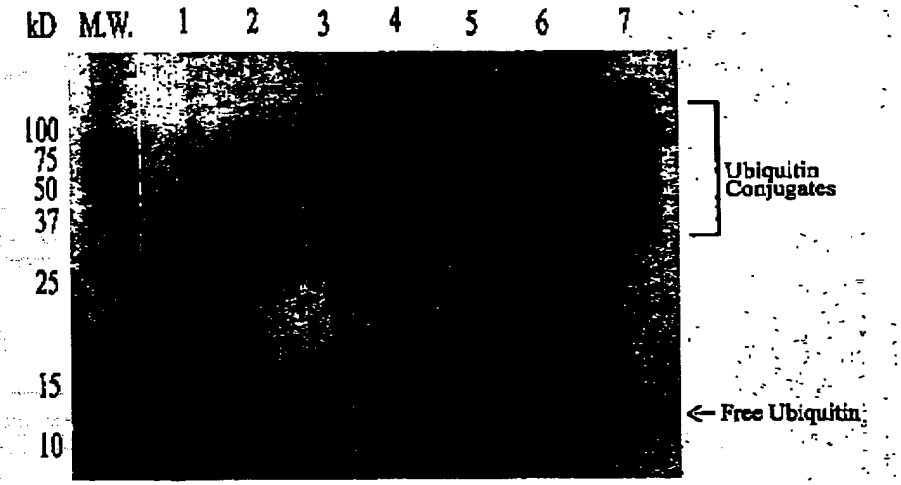
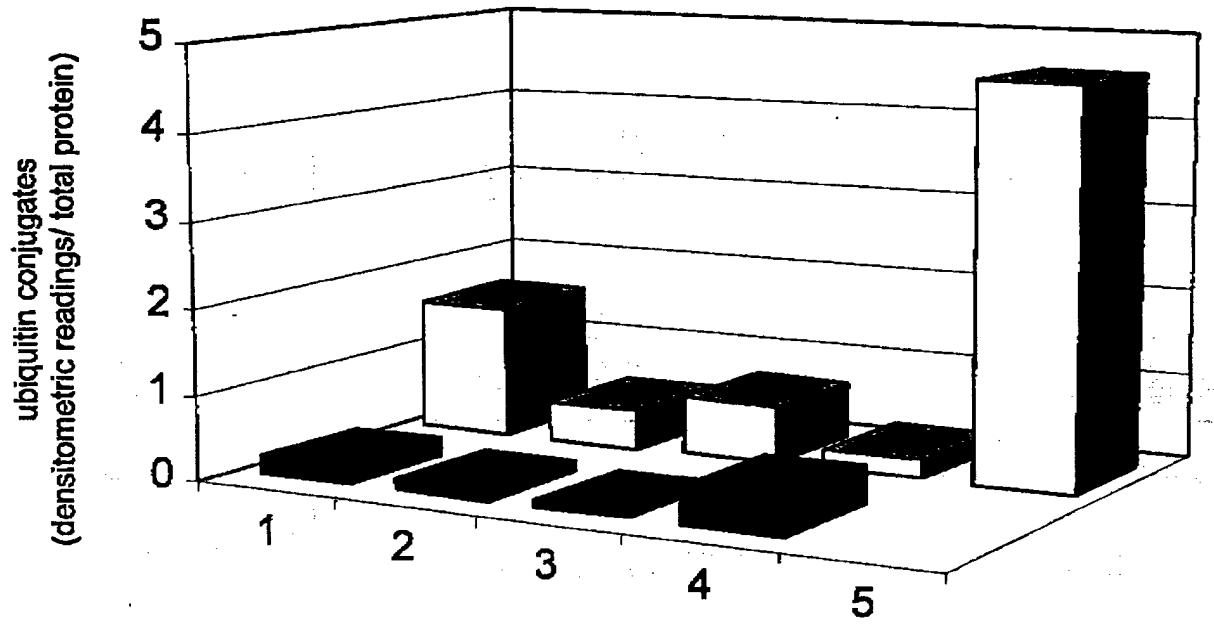

kD
M.W.
1
2
3
4
5
6
7
100
75
50
37
25
15
10
Ubiquitin Conjugates
← Free Ubiquitin

FIGURE 3

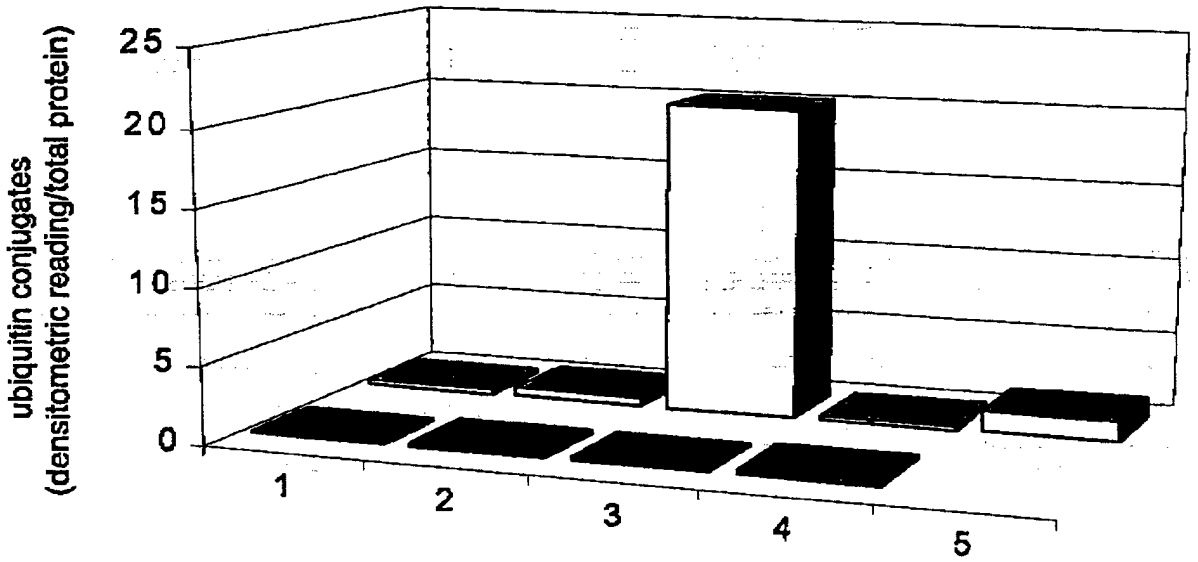
Ubiquitin Conjugates in Mouse Hamstring Muscle



animal number	1	2	3	4	5
■ non-tumor-bearing	0.2175	0.1306	0.0757	0.4385	
□ tumor-bearing	1.5588	0.4601	0.6733	0.2172	4.5891

FIGURE 4

Ubiquitin Conjugates in Mouse Paraspinal Muscle



animal number	1	2	3	4	5
■ not tumor-bearing	0.1512	0.2222	0.2378	0.3021	
□ tumor-bearing	0.3272	0.5582	20.295	0.2288	1.408

Restoration of fertility in young obese (*Lep*^{ob}*Lep*^{ob}) male mice with low dose recombinant mouse leptin treatment

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Running head: **Leptin treatment in obese male mice**

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We investigated the effects of low dose leptin treatment on restoration of fertility in young adult male leptin deficient obese mice. 8-10 wk old MMTV-TGF-α *Lep^{ob}Lep^{ob}* mice were treated with recombinant mouse leptin. In Experiment 1, four mice (5ug/g bw leptin followed by 2.5ug/g) lost weight and impregnated females (# pregnancies/# females, 3/6,5/6,5/10,4/10). In Experiment 2, LEPTIN-OBESE (2.5ug/g) and CONTROL-LEAN mice weighed significantly less than CONTROL-OBESE mice. Epididymal pad weights of CONTROL-OBESE mice were the heaviest followed by those of LEPTIN-OBESE mice, and CONTROL-LEAN mice had the lightest. Testes weight was greater in CONTROL-LEAN versus CONTROL-OBESE mice. LEPTIN-OBESE mice had testes weight not significantly different from either control group. Four of five LEPTIN-OBESE mice impregnated females (4/10,5/10,2/10,5/12,0/10). These results indicate that low dosage mouse recombinant leptin treatment restored fertility to young *Lep^{ob}Lep^{ob}* male mice. Although body weights of LEPTIN-OBESE mice were similar to those of lean age-matched mice, epididymal fat pad weights were heavier.

Keywords: leptin, obese, male mice, fertility, *Lep^{ob}Lep^{ob}*

Introduction

Mice recessive for *Lep*^{ob} are obese, hyperinsulinemic, hypertriglyceridemic and infertile (1;2). Leptin treatment of *Lep*^{ob}*Lep*^{ob} mice prevents weight gain or causes weight loss dependent upon the age and leptin dosage (3-9). Recent studies reported that treatment of obese *Lep*^{ob}*Lep*^{ob} mice with recombinant human leptin restored fertility (10;11). In adult male mice, ~70g, daily intraperitoneal (IP) injection with 20μg/g body weight recombinant human leptin resulted in 5/5 mice impregnating females (11). Lower leptin doses result in weight loss in *Lep*^{ob}*Lep*^{ob} mice (5;12;13), and thus might also restore fertility. We evaluated the effect of lower leptin dosages on fertility in young male obese MMTV-TGF-α/*Lep*^{ob}*Lep*^{ob} mice. Female MMTV-TGF-α mice are at increased risk to develop mammary tumors (14). It was anticipated that restoration of fertility in obese male mice would enhance production of offspring needed to conduct experiments evaluating the role of obesity on tumor development.

Experimental design and results

Mice were obtained from a colony of MMTV-TGF-α/*Lep*^{ob} C57BL/6J mice established at the Hormel Institute. The study was approved by the University of Minnesota Institutional Animal Care and Use Committee and the facility is AAALAC accredited. Mice were maintained in a temperature- and humidity-controlled room with 12h light:12h dark cycle, fed commercial rodent food, and supplied with water. Mice were genotyped to determine TGF-α status and whether they were *Lep*$^{+}$*Lep*$^{+}$, *Lep*$^{+}$*Lep*^{ob}, or *Lep*^{ob}*Lep*^{ob}. Obese 8-10 week old mice were used weighing approximately 43 g. Recombinant mouse leptin (PeproTech, Rock Hill, NJ) was dissolved in 5 mM Tris Buffer, pH 8.0, diluted in

phosphate buffered saline, and pH adjusted to 7.1-7.2 for a final concentration of 0.5 μg/μl. Leptin was administered between 8-10 A.M.

In **Experiment 1** TGF-α/*Lep*^{ob}*Lep*^{ob} male mice (n=4) were treated daily (5μg/g body weight leptin IP injections) for 10-16 days followed by 2.5μg/g for 7-18 more days. After one week, males were housed with 1-2 female *Lep*$^{+}$*Lep*^{ob} mice for 5-7 days. Female mice were removed, placed individually in whelping cages, and observed for offspring production. Data are presented as mean±SEM and were analyzed by paired Student's *t* test.

Leptin-treated *Lep*^{ob}*Lep*^{ob} obese male mice weighed significantly less at the end of treatment in comparison to their initial weights (Figure 1). All four *Lep*^{ob}*Lep*^{ob} male mice proved fertile with 3/6, 5/6, 5/10, 4/10 female mice producing offspring. Forty-eight of ninety pups were *Lep*^{ob}*Lep*^{ob}.

In **Experiment 2,** five TGF-α/*Lep*^{ob}*Lep*^{ob} **LEPTIN-OBESE** mice were treated daily with 2.5 μg/g body weight leptin IP for 34-38 days. **CONTROL-OBESE** mice (n=6) received vehicle injections. Following 7-10 days, **LEPTIN-OBESE** and **CONTROL-OBESE** mice were housed weekly with pairs of female *Lep*$^{+}$*Lep*^{ob} lean mice. Females were removed, placed in whelping cages, and observed to determine offspring production. When mice were killed, testes, seminal vesicles, and epididymal pads were removed and weighed. Also, **CONTROL-LEAN** (*Lep*$^{+}$*Lep*$^{+}$) male mice (n=6) matched for age were killed. Data are presented as mean±SEM and were analyzed by ANOVA. When the F value was significant, post hoc analysis was by Newman-Keuls test.

Body weight of **LEPTIN-OBESE** male mice was significantly lower than that of **CONTROL-OBESE** mice and similar to that of **CONTROL-LEAN** mice (Table 1). Epididymal pad weight of **LEPTIN-OBESE** mice was 70% lower then that of **CONTROL-OBESE** mice, but pad weight of **CONTROL-LEAN** mice was significantly lighter than both obese groups (Table 1). Testes weights of **CONTROL-OBESE** mice were significantly lower than those of **CONTROL-LEAN** mice, while **LEPTIN-OBESE** mice had intermediate testes weight. Seminal vesicle weights were similar among the three groups. Epididymal pad, testes and seminal vesicles weights mg/g body weight were greatest in **CONTROL-LEAN**, intermediate in **LEPTIN-OBESE** and lowest in **CONTROL-OBESE** mice (Table 1).

Four **LEPTIN-OBESE** mice produced offspring, *i.e.*, 4/10, 5/10, 2/10, 5/12, 0/10 females became pregnant. No pregnancies resulted from the first encounter suggesting that 10-14 days treatment was required before fertility was affected. Forty-two of seventy-six weaned pups were $Lep^{ob}Lep^{ob}$. There were no pregnancies in 44 females housed with four **CONTROL-OBESE** mice. Two **CONTROL-OBESE** and the **LEAN-CONTROL** mice were not mated due to limitations of available female mice.

Discussion

These results extend the earlier report that 20 μg/g body weight leptin restored fertility in mature male $Lep^{ob}Lep^{ob}$ mice (11). We found that doses of mouse recombinant leptin 6-8 times lower than previously used were effective in restoration of fertility in younger lighter male $Lep^{ob}Lep^{ob}$ mice. Given the high cost of leptin this has important impact for those wanting to restore fertility to these mice.

Although low dose leptin treatment resulted in pregnancies in 8 of 9 treated mice (compared to 36/37 TGF-α lean mice mated during this time), the rate of pregnancies appeared to be less than that reported for the 20μg/g body weight dose. However, the 5 mice in the earlier study (11) were each exposed to only two females. Thus, it is difficult to make direct comparisons.

Visibly **LEPTIN-OBESE** mice were indistinguishable from **CONTROL-LEAN** mice. Despite this and their similar body weights, they had significantly elevated epididymal pad weights compared to age-matched **CONTROL-LEAN** mice. Previously, more body fat was reported for leptin-treated obese mice than for lean mice (4;15). However, those obese leptin-treated mice weighed significanlty more than the lean mice.

Leptin's effects on reproduction are thought to be mediated through the hypothalamus (16). However, small amounts of leptin may enter the testes by a nonsaturable process (17). In addition, a recent *in vitro* study suggested that age may play a role in testicular response to leptin (18).

In conclusion, fertility was restored in young male obese $Lep^{ob}Lep^{ob}$ mice using low dosage recombinant mouse leptin. However, the success rate for obtaining pregnancies appears to be lower than that reported for higher doses of human recombinant leptin in older obese mice. Given the short half-life and pulsatile nature of leptin secretion (19), a different mode of administration of leptin resulting in a consistent blood level may result in higher pregnancy rates. Furthermore, although body weights of leptin-treated obese mice were similar to those of age-matched lean mice, epididymal pad weights were still heavier.

Acknowledgments

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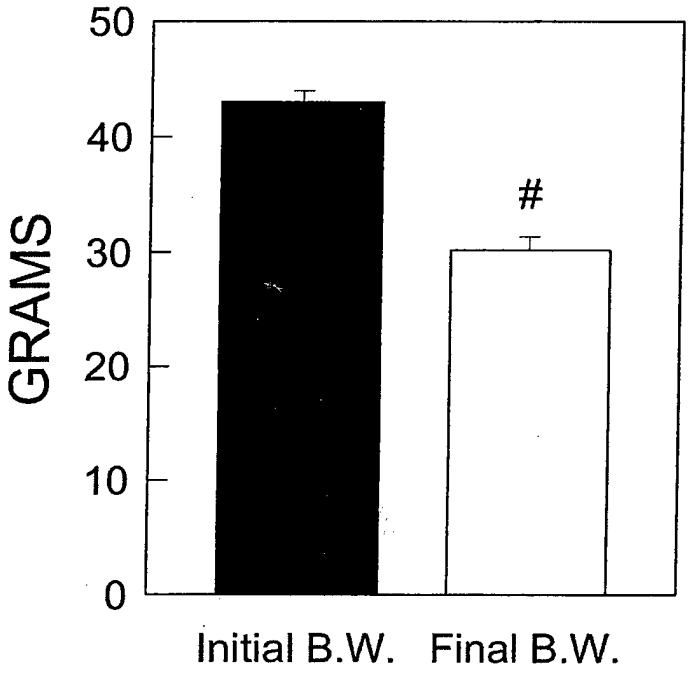
Table 1 (Experiment 2) Body, epididymal fat pad, testes, and seminal vesicles weights of **LEPTIN-OBESE** (n=5), **CONTROL-OBESE** (n=6), and **CONTROL-LEAN** (n=6) mice.

	LEPTIN-OBESE	CONTROL-OBESE	CONTROL-LEAN
		Weights (g)	
Body Weight	$29.56^{a} \pm 0.83$	$56.76^{b} \pm 2.16$	$26.01^{a} \pm 0.729$
Epididymal Fat Pad	$1.170^{b} \pm 0.117$	$3.649^{c} \pm 0.337$	$0.237^{a} \pm 0.044$
Testes	$0.223^{a} \pm 0.013$	$0.200^{a} \pm 0.020$	$0.264^{b} \pm 0.014$
Seminal Vesicles	$0.210^{a} \pm 0.028$	$0.259^{a} \pm 0.055$	$0.249^{a} \pm 0.010$
		Organ weights per body weight (mg/g)	
Epididymal Fat Pad	$37.15^{b} \pm 4.31$	$64.43^{c} \pm 5.74$	$8.944^{a} \pm 1.42$
Testes	$7.52^{b} \pm 0.34$	$3.52^{a} \pm 0.32$	$10.11^{c} \pm 0.33$
Seminal Vesicles	$7.05^{b} \pm 0.80$	$4.56^{a} \pm 0.94$	$9.56^{c} \pm 0.35$

LEPTIN-OBESE mice treated with 2.5 μg/g recombinant mouse leptin for 34-38 days. Data are presented as mean ± SEM. Data analyzed by ANOVA followed by Neuman-Keuls post hoc test. Numbers in rows with different superscripts are significantly different.

FIGURE 1



FINAL REPORT BIBLIOGRAPHY AND LIST OF PERSONNEL:

Publications

Jatoi, A., M.P. Cleary, C.-M. Tee, and P.L. Nguyen. Weight gain does not preclude activation of the ubiquitin-proteasome system in skeletal muscle of tumor-bearing mice. Annals of Nutrition and Metabolism (accepted pending revision). (Appendix N)

Cleary, M.P., H.M. Bergstrom, T.L. Dodge, S.C. Getzin, M.K. Jacobson, and F.C. Phillips. Restoration of fertility in young obese ($Lep^{ob}Lep^{ob}$) male mice with low dose recombinant mouse leptin treatment. International Journal of Obesity (in press). (Appendix O)

Abstracts, invited speaker and poster presentations

Invited speaker, "An Animal Model to Study the Effect of Obesity on the Development of Mammary Tumors," Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN October 1998

Invited speaker, "Development of an Animal Model to Investigate the Relationship Between Body Weight and Breast Cancer," Department of Food Science and Nutrition, University of Minnesota, St. Paul, MN December 1998.

Invited speaker, "Body Weight and the Development of Breast Cancer," Austin Rotary Club, Austin, MN March 1999.

Poster presentation, "Development of a New Mouse Model to Study the Effect of Obesity on the Development of Mammary Tumors," Toxicology Society Symposium – Role of Diet and Caloric Intake in Aging, Obesity, and Cancer, Reston, VA October 1998.

Poster presentation, "Restoration of Fertility in Young Adult Male TGF-α $Lep^{ob}Lep^{ob}$ Hybrid Mice with Lose Dose Leptin Treatment," Experimental Biology, Washington, D.C. April 1999 (*FASEB J.* 13, A927, 1999).

Invited speaker, "The Effect of Obesity on the Development of Mammary Tumors," Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN October 1999

Invited speaker, "Body Weight and the Development of Breast Cancer," Lyle Eagles, Lyle, MN April 1999.

Poster presentation, "TGF-α/$Lep^{+}Lep^{ob}$ lean mice weigh more and have higher mammary tumor (MT) incidence than do TGF-α/$Lep^{+}Lep^{+}$ lean mice" 91st American Association for Cancer Research meeting, San Francisco, CA, April 2000. (*Proc. Am. Assoc. Cancer Res.* 41, 83-84, 2000).

Poster presentation, "Weight gain does not preclude activation of the ubiquitin-proteasome system in skeletal muscle of tumor-bearing mice". 91st American Association for Cancer Research meeting, San Francisco, CA, April 2000. (*Proc. Am. Assoc. Cancer Res.* 41, 200, 2000).

Poster presentation, "Weight-cycling of TGF-α/*Lep*$^{+}$*Lep*^{ob} female mice: effects on oncogene-induced mammary tumors (MTs), body weight, and food intake". Experimental Biology meeting San Diego, CA, April 2000.

Invited talk, "Elevated body and carcass weights are associated with a higher incidence of proto-oncogene induced mammary tumors in MMTV-TGF-α mice". DOD Era of Hope meeting, Atlanta, GA, June 2000.

Poster presentation, "Increased incidence of mammary tumors in MMTV-TGF-α/*Lep*$^{+}$*Lep*$^{+}$ obesity-prone mice in comparison to MMTV-TGF-α/*Lep*$^{+}$*Lep*$^{+}$ obesity-resistant mice". DOD Era of Hope meeting, Atlanta, GA, June 2000.

Poster presentation, "Elevated body and carcass weights are associated with a higher incidence of proto-oncogene induced mammary tumors in MMTV-TGF-α mice". DOD Era of Hope meeting, Atlanta, GA, June 2000.

Personnel receiving pay from the research effort

Margot P. Cleary
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